SUPPLEMENTARY METHODS

Cell Culture

The Kasumi-1, HL-60, and U937 cell lines were purchased from the American Type Culture Collection (ATCC). Cells were cultured in RPMI 1640 (Cellgro) with 10% fetal calf serum (FCS) (Sigma-Aldrich) and 1% penicillin-streptomycin. SKNO1 cells were obtained from the laboratory of Jonathan Licht and cultured in RPMI 1640 with 20% FCS, 1% penicillin-streptomycin, and 1 ng/ml GMCSF (BD Biosciences).

RNA Interference

The siRNA constructs against AML1-ETO and firefly luciferase were designed as previously described.[1] The AML1-ETO-directed siRNA contained CCUCGAAAUCGUACUGAGAdTdT as the sense strand and UCUCAGUACGAUUUCGAGGdTdT as the antisense strand. The control firefly luciferase (siGL)-directed siRNA contained CGUACGCGGAAUACUUCGAdTdT as the sense strand and UCGAAGUAUUCGGCGUACGdTdT as the antisense strand. RNA sequences were synthesized by Dharmacon. Lyophilized siRNA was resuspended in 1x siRNA buffer (20 mM KCl, 6 mM HEPES pH 7.5, 0.02 mM MgCl2) and aliquoted for storage at a final concentration of 75 μM. Cells were transfected either by Amaxa nucleofection or by siLentFect lipid-based reagent (Biorad). Amaxa transfection was performed using 2 μl siRNA in 100 μl Amaxa buffer V (final siRNA concentration 1.5 μM) and program P-19 per the manufacturer's protocol. For the cells transfected by lipid reagent, siRNA was diluted into serum-free medium at a final concentration of 240 nM and incubated with siLentFect (1.5 μl per 25 μl medium) for 20 minutes at room

temperature before addition to 500,000 Kasumi-1 cells per well in 500 µl medium. Following transfection, cells were incubated at 37° C for 72 or 96 hours. Knockdown of AML1-ETO was confirmed by immunoblot and real-time PCR.

Immunoblotting

Cells were lysed in RIPA buffer (10 mM Tris-Cl pH 7.6, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) with protease inhibitor (Complete Mini EDTA free protease inhibitor tablets, Roche Diagnostics), resolved by electrophoresis on 10% Tris-HCl pre-cast Ready Gel (BioRad Laboratories), and transferred to PVDF membranes (Millipore). Blots were blocked with milk for 30 minutes, washed in 1x tris-buffered saline containing Tween-20 (TBS-T), and probed with total AML1 rabbit polyclonal IgG antibody (PC285, Calbiochem), total ETO rabbit polyclonal IgG antibody (PC283, Calbiochem), or pan-actin antibody (ACTN05, NeoMarkers). Blots were rinsed and probed with anti-rabbit-HRP (Amersham #NA9340V). Bound antibody was detected by chemiluminescence.

Real-time RT-PCR

Total RNA was isolated at 24 hours using TRIZOL reagent (Invitrogen) from Kasumi-1 cells transfected with AML1-ETO-directed siRNA or the control siGL siRNA. cDNA was synthesized from 1 µg of total RNA using SuperScript III Reverse Transcriptase (Invitrogen) and oligo $d(T)_{16}$ primers in a 20 µL reaction system. One µL of 1:6 diluted cDNA was analyzed in the real-time quantitative PCR reactions prepared with TaqMan

Universal Master Mix (Applied Biosystems). Each sample was assessed in triplicate to ensure reproducibility of the quantitative measurements. GAPD or Abl expression was evaluated for each sample as a control for total RNA. Primers and probes for real-time RT-PCR were obtained from Applied Biosystems (GAPD #402869, Abl #Hs99999002_mH, and AML1-ETO assay by design #191284).

Expression Profiling

Kasumi-1 cells were transfected in triplicate with AML1-ETO or luciferase siRNA constructs by either Amaxa nucleofection or Biorad siLentFect and incubated for 96 hours. RNA was extracted with TRIZOL per the manufacturer's guidelines, and 10 μg was used to create microarray target samples. First strand cDNA synthesis was generated using a T7-linked oligo-dT primer, followed by second strand synthesis. An in vitro transcription reaction was performed to generate cRNA containing biotinylated UTP and CTP, which was subsequently chemically fragmented at 95°C for 35 minutes. Ten micrograms of the fragmented, biotinylated cRNA was hybridized in MES buffer (2-[N-Morpholino]ethansulfonic acid) containing 0.5 mg/ml acetylated bovine serum albumin (Sigma) to Affymetrix U133A arrays at 45°C for 16 hours. U133A arrays contain approximately 22,283 probe sets that correspond to known genes and expressed sequence tags. Arrays were washed and stained with streptavidin-phycoerythrin (SAPE, Molecular Probes). Signal amplification was performed using a biotinylated anti-streptavidin antibody (Vector Laboratories) at 3 μg/ml. This was followed by a second staining with SAPE. Normal goat IgG (2 mg/ml) was used as a blocking agent. Scans were performed

on Affymetrix scanners. Raw microarray data are available at http://www.broad.mit.edu/cancer/pub/AML1-ETO_GE-HTS

Marker Gene Selection

GeneChip MAS5 Software was used for preprocessing of the raw expression data, and all scans within an experiment were scaled to the array with the median overall microarray intensity. Thresholds were set to a minimum value of ten and a maximum value of 16,000, and a variation filter of three-fold minimum and 100 unit minimum absolute difference was applied. We used the signal-to-noise ratio (SNR) to rank order the genes that distinguish "AML1-ETO positive" from "AML1-ETO knockdown" and then permutation testing (1,000 permutations) to identify genes that distinguish these states with $p < 0.05$. Next, genes were identified with at least a three-fold difference between the two classes of interest. In addition, the expression of these genes was evaluated in an AML1-ETO-inducible dataset obtained from U937 cells expressing an inducible AML-ETO construct under the control of the tetracycline promoter.[2] These cells were profiled on Affymetrix U95Av2 oligonucleotide microarrays. Samples were collected at various times after induction. Control samples at 0, 12, 24, 48, and 72 hours were compared to AML1-ETO induced samples at 48 and 72 hours (2 samples at each time point). Through a comparison of the stable knockdown and inducible rescue datasets, we selected 24 genes that were well-correlated with AML1-ETO expression in both datasets and had $p < 0.05$ in at least one of the data sets. One additional gene, *bactericidal permeability-increasing protein (BPI)*, was included despite inconsistent expression in the U937 data given evidence in the literature to support its regulation by AML1.[3] *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPD*), *beta-actin (BACT)*, and *RPL0* were included as

controls for well-to-well variability, as these genes demonstrated a stable expression pattern in all conditions. A probe for wild-type AML1 was also included as a control although it was not used in the final analysis.

Small Molecule Library Screening Methods

Cell and compound addition

Kasumi-1 cells were plated in 384-well tissue culture plates in 50 μl of medium at 25,000 cells/well using the Multidrop 384 instrument (Thermo). The following controls were included on each 384-well screen plate: medium only (16 wells), Kasumi-1 cells (16 wells), AML1-ETO knockdown RNA (8 wells), and Kasumi-1 control RNA (8 wells).

Compounds were added at a final approximate concentration of 20 μM in DMSO by pin transfer and incubated for 72 hours at 37°C with 5 % CO_2. We screened a total of 2,480 compounds in triplicate, including the National Institute of Neurological Disorders and Stroke (NINDS) small molecule collection (1040 compounds), the SpecPlus collection (960 compounds), and the BIOMOL ICCB known bioactives collection (480 compounds). Compounds contained within each library are available online at: http://broad.harvard.edu/chembio/platform/screening/compound_libraries/index.htm. Three-quarters of the compounds in the NINDS collection of characterized bioactive molecules are FDA-approved.

RNA extraction and reverse transcription

After 72 hours of chemical incubation, 25 μl of lysis buffer was added to the wells with a Multimek robot (Beckman Coulter). Cells were then lysed for 30 minutes at room

temperature. Lysate was transferred to a 384-well oligo-dT-coated plate and incubated for one hour at room temperature. Reverse transcription was carried out in a 5 μl MMLV reaction (Promega) for two hours at 37°C. Lysis buffers and 384-well oligo-dT plates were purchased from RNAture (Qiagen).

Ligation-mediated amplification (LMA)

Custom LMA gene probes were designed for each signature gene. Gene-specific sequences were chosen such that the 20 base pair sequences of the upstream and downstream probes contain similar base composition, minimal repeats, and C-G or G-C juxtaposing nucleotides (Supplementary Table 1). Where possible, at least two separate probes were designed and tested for each gene for their ability to discriminate between RNA from Kasumi-1 cells with and without AML1-ETO knockdown. The top-performing pilot probes for each gene were selected to comprise the final screen probe set. Following cDNA synthesis, solution was spun out. Signature gene-specific oligonucleotide probes were hybridized to the cDNA using 2 nM of each probe (16 probe pairs in total) and Taq ligase buffer (New England Biolabs) in 5 μl total volume. Upstream probes contained the T7 primer site, Luminex designed FlexMap barcode tag (24nt), and gene-specific sequence (20nt). Downstream probes were phosphorylated at the 5' end and contained a gene-specific sequence (20nt) followed by the T3 primer site. Hybridization was performed at 95°C for two minutes followed by 50°C for one hour. Excess probe was then spun out. Probes were ligated in a 5 μl reaction using Taq DNA ligase (New England Biolabs) at 45°C for one hour followed by incubation at 65°C for 10 minutes. Excess ligation mix was spun out. The ligated products were amplified with the

universal T3 primer (5'-ATT AAC CCT CAC TAA AGG GA-3') and universal biotinylated T7 primer (5'-TAA TAC GAC TCA CTA TAG GG-3') (Integrated DNA Technologies) using HotStarTaq DNA Polymerase (Qiagen) in a 5 μl reaction system. PCR was performed at 92°C for nine minutes, followed by 34 cycles of denaturation at 92°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds.

Amplicon detection

xMAP multi-analyte COOH microspheres (Luminex) (2.5 million) were coupled to complementary FlexMap barcode sequence (4 μM) with 2.5 μl of 10 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (Pierce) in 25 μl 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) pH 4.5 buffer (Sigma). This 30-minute coupling reaction was then repeated. Microspheres were then washed sequentially with 0.02% Tween-20, 0.1% SDS, and TE (pH 8) and resuspended in 50 μl TE (pH 8). Next, LMA sample was hybridized to microspheres by incubating 2,500 of each microsphere in 18 μl of 1.5X TMAC (4.5 M tetramethylammonium chloride, 0.15% N-lauryl sarcosine, 75mM Tris-HCl (pH 8), and 6mM EDTA (pH 8)) and 5 μl of TE (pH 8) at 95°C for two minutes and then 45°C for one hour. For detection, the sample was incubated with 10 μl of streptavidin-phycoerythrin (SA-PE) (Molecular Probes) (1% SA-PE in 1x TMAC (3M tetramethylammonium chloride, 0.1% N-lauryl sarcosine, 50 mM Tris-HCl (pH 8), and 4mM EDTA (pH 8)) for five minutes at 45°C and then washed once and resuspended with 1x TMAC. Dual-color fluorescence was detected with a Luminex high-throughput

detection instrument. A minimum of 100 events were recorded for each microsphere and median fluorescent intensities (MFI) computed.

Hit identification

The median fluorescence intensity for each gene (represented by an individual bead color) from the Luminex detector was used as a measure of the gene's expression. To maximize consistency between and within plates, we normalized genes by using the expression ratio between each gene and an average of three control genes (*GAPD*, *beta-actin*, and *RPL0*). Next, filtering was performed to eliminate "dead" wells from further analysis where *GAPD* expression levels were used as a proxy for cell viability. Because ratios with control genes in the denominator were used for subsequent analysis, it was necessary to eliminate wells where the control genes were nominally zero. Forming a ratio with such a low denominator could potentially falsely flag a well as a hit when the cells were merely dying. The average across the screen of the *GAPD* background intensity in the medium-only wells was used as the filtering threshold. After filtering, each plate was median scaled to reduce plate-to-plate variation. Median scaling sets the median of the ratio values for the positive controls (AML1-ETO knockdown RNA) on each plate for each gene ratio equal to one. Compounds that induce the AML1-ETO abrogation signature were identified using three discrete analytic metrics: weighted summed score, k-nearest neighbor (KNN), and naïve Bayes classification (see below). Chemical hits for follow-up were selected from compounds that induced the AML1-ETO signature according to at least two of the three analytic metrics (at a probability greater

than 0.9 for weighted summed score or naïve Bayes or all three replicates were classified as AML1-ETO signature abrogation by KNN).

The summed score metric combined expression ratios by summing them with a sign determined by the expected direction of regulation as determined from the positive controls. The weighted summed score metric is a variant of the summed score metric that combines expression ratios by summing them with a weight and sign determined by the signal-to-noise ratio of the positive control (AML1-ETO knockdown) and negative controls (Kasumi-1 controls). Signal-to-noise ratio is defined by:

$$W_i = \frac{\mu_{i1} - \mu_{i2}}{\sigma_{i1} + \sigma_{i2}}$$

where μ_{i1} represents the mean expression of samples from class 1 for feature i and σ_{i1} represents the standard deviation of class 1 for feature i.[4] Composite scores for both the summed score and weighted summed score were formed by finding the average of the scores from the three replicates.

Each compound's summed score and weighted summed score was assigned a probability that the compound caused the cells to have an expression signature like those for the AML1-ETO knockdown control wells. The calculation of the probability was based upon finding the Bayesian probability density of the score using normal distributions to model each of two classes of controls:

$$p(C = c \mid X = x) = \frac{p(C = c)\,p(X = x \mid C = c)}{p(X = x)}, \text{ where}$$
$$p(X = x \mid C = c) = N(x; \mu_c, \sigma_c)$$

$N(x;\mu_c,\sigma_c)$ was the probability density function for a normal (or Gaussian) distribution with mean μ_c and standard deviation σ_c.[5] The parameters for the Gaussian distribution were trained on the positive and negative controls and $p(C=c)$ was the *a priori* probability of class c controls (in this case, we assumed the positive and negative controls have equal *a priori* probabilities). Composite probabilities were found by taking the product of the probabilities for the three replicates (but leaving out filtered replicates) and renormalizing the probabilities to ensure that the probability that the compound is a positive control and the probability that the compound is a negative control sum to one. Compounds were ranked for follow-up according to the probability that they looked like the AML1-ETO knockdown positive control.

A Naïve Bayes classifier was also used to evaluate the expression signatures for the compounds. The Naïve Bayes classifier is based upon the Bayes probability rule and naïvely assumes that the features are independent within each class. The independence assumption greatly simplifies the calculation of the class probabilities and has been shown to work well even in cases where the features have significant dependencies. The probabilities are calculated as follows:

$$p(C = c \mid \mathbf{X} = \mathbf{x}) = \frac{p(C = c)\, p(\mathbf{X} = \mathbf{x} \mid C = c)}{p(\mathbf{X} = \mathbf{x})}, \text{ where}$$

$$p(\mathbf{X} = \mathbf{x} \mid C = c) = \prod_i p(X_i = x_i \mid C = c)$$

for continuous values like the gene expression ratios $p(X_i=x_i|C=c)$ were a kernel distribution formed out of a mixture of Gaussians.[6] The parameters for the distribution for each class c and each feature i are trained using the controls for the screen. The overall probability for each compound is found by multiplying the probabilities for the

individual replicates (leaving out filtered replicates) and renormalizing the probabilities so the two classes to sum to one. Compounds were ranked for follow-up according to the probability that they looked like the AML1-ETO knockdown RNA positive control wells.

The k-nearest-neighbor (KNN) classifier was also used to identify possible hits. It assigns samples to the class most frequently represented among the k nearest control samples.[5] A KNN predictor was trained using the positive control (AML1-ETO knockdown RNA wells) and negative control (Kasumi-1 control RNA wells) samples and the compound treated wells were tested using k=3 with a Pearson correlation for the distance metric and weights for the features based upon their signal-to-noise ratio in the control samples.

May-Grunwald Giemsa Staining

Kasumi-1 cells were treated with indicated concentrations of chemicals for 72 hours and spun onto glass slides using Cytospin 4 (Thermo). Cells were fixed in methanol for five minutes and stained with May-Grunwald solution (Sigma MG80) for five minutes. Following a five-minute PBS wash, cells were stained with 1:20 modified Giemsa stain (Sigma GS1L) for twenty minutes and then rinsed with tap water. Images were acquired by Q-Color 5 camera (Olympus) under oil.

Flow Cytometry

For myeloid surface marker evaluation, 400,000 Kasumi-1 cells were plated in triplicate with DMSO vehicle, 80 or 160 nM methotrexate or methylprednisolone (Sigma) and

incubated for 72 hours. Cells were washed in 1x PBS/1% FCS and incubated with 1:25 CD11b-FITC (Beckman Coulter IOTest IM0530U) and 1:25 CD14-PE (Beckman Coulter IOTest IM0650U) for 30 minutes. One replicate for each compound was left unstained in order to rule out autofluorescence associated with cell death. Cells were washed with 1x PBS/1% FCS, resuspended in 300 μl 1x PBS and detected by flow cytometry (Beckman Cytomics FC500). Data was analyzed using the FlowJo software package (Tree Star). Fluorescence gating was set based on single-stained mouse Ig κ compensation bead fluorescence intensity (BD Biosciences #552843).

Apoptosis studies were performed with Kasumi-1 cells treated with DMSO versus methotrexate (40, 80 and 160 nM) or methylprednisolone (40, 80 and 160 nM) for 3 days. Annexin V FITC/PI staining was performed with the Annexin V: FITC Apoptosis Detection Kit I (BD Pharmingen). Cells were analyzed by flow cytometry with a FACScan flow cytometer (Becton Dickinson) and CELLQuest analytical software.

Myeloid Differentiation Signature

Marker genes for myeloid differentiation were chosen using previously published Affymetrix data sets containing primary AML cells, normal human neutrophils, normal human monocytes, and the HL-60 AML cell line differentiated with either ATRA, phorbol 12-myristate 13-acetate (PMA), or 1,25-dihydroxyitamin D3 (vitD).[7] We prioritized a 32-gene signature containing genes that distinguish AML from either neutrophil or monocyte with $p < 0.05$ by t-test. These genes also distinguished

undifferentiated versus differentiated HL-60 with ATRA, PMA, or VitD with $p < 0.05$ by t-test. Probes are shown in Supplementary Table 2.

Viability Assay

Viability experiments were performed in 96-well format in replicate of four using the Promega Cell-Titer Glo ATP-based assay per the manufacturer's instructions. Kasumi-1 cells were evaluated at 72 hours with hit compounds in a two-fold dilution to establish the drug concentrations that reduced cell viability to 50 percent of the vehicle controls (IC50). For comparison purposes, IC50 values were also obtained for hit compounds in the AML1-ETO-negative HL-60, and U937 cell lines. Values for IC50 were calculated by interpolating a natural cubic spline fit to the measured viability data in R (using the spline function). The natural spline requires that the second derivative of the interpolated curve equal zero at the endpoints of the interval of interpolation. The IC50 value was found by evaluating in R the interpolated spline at 0.5 using the approx function.

References

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